

**INTERNATIONAL JOURNAL OF SCIENCE FOR GLOBAL SUSTAINABILITY****Optimization of Bioethanol Production from Sugar Bagasse by
Aspergillus flavus Using Box Behnken Design**Muhammad Surajo Umar^{1,*}, Auwalu Muttaka¹, Abdulwahid Abdullahi Naibi¹, Musa, A.U.² and K. Kabir³¹Department of Biochemistry, Faculty of Sciences, Federal University Gusau, P.M.B 1001, Gusau, Nigeria.²Kano Bioresources Center, National Biotechnology Development Agency, Nigeria³Department of Chemistry, Faculty of Sciences, Nigerian Defense Academy Kaduna, Nigeria.*Corresponding Author's E-mail: muhdsuraj@fugusau.edu.ng

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ABSTRACT

"Bioethanol, a renewable fuel derived from biomass feedstocks like agricultural residues and forestry waste, holds immense promise for sustainable energy production. Notably, sugar bagasse—a residual lignocellulosic material after sugarcane juice extraction—offers a valuable substrate due to its high sugar content. To harness its potential for bioethanol generation, optimizing parameters, such as temperature, pH, and fermentation duration. This study is geared towards the strategic enhancement of bioethanol synthesis from sugar bagasse, employing the Box Behnken experimental design using *Aspergillus flavus* as the biocatalyst. Employing rigorous statistical modeling and analysis, the investigation dissects the intricate interplay of temperature, pH, and fermentation duration on the bioethanol production kinetics. The outcomes underscore the optimal operational nexus that attains peak bioethanol yield: a thermal setpoint of 32.6°C, a pH milieu of 5.3, and a fermentation interval spanning 57.9 hours. The discerned synergy of these conditions culminates in an impressive bioethanol yield of 41.6 mg/L, manifesting a remarkable 56.9% escalation relative to the baseline conditions. This inquiry not only imparts nuanced insights into the precision-tuning of bioethanol synthesis from sugar bagasse through *Aspergillus flavus* but also furnishes a pivotal trajectory for the advancement of sustainable, environmentally conscious biofuel production."

Keywords: bioethanol, *Aspergillus flavus*, feedstock, sugarcane bagasse, box behnken.**1.0 Introduction**

The vulnerability of civilization to global warming is unquestionably one of the most complex environmental issues of nowadays (Sun *et al.*, 2023). This trajectory has continued with global energy consumption tripling since the year 2000 and a projected ascent to 740 million terajoules by 2040, as forecasted by the International Energy Agency (Owusu & Asumadu-Sarkodie, 2016). Yet, this ongoing dependency on non-renewable and ecologically detrimental carbon sources, namely petroleum, coal, and natural gas, has posed a substantial existential threat to the planet due to their contribution to global warming (Johnsson *et al.*, 2019). In response to this predicament, a pressing demand exists to identify alternative and sustainable energy sources capable of diminishing

CO₂ emissions and alleviating the repercussions of climate change.

Among these alternatives, biofuels hold considerable promise as a renewable energy reservoir, originating from organic matter and applicable in both transportation and electricity generation contexts to overcome current environmental crisis (Santika *et al.*, 2019). Recent times have witnessed a surging trend in the adoption of renewable fuels—such as biodiesel, biogas, and bioethanol—as substitutes for traditional fossil fuels. Although bioethanol, a product predominantly derived from edible biomass sources like beets, sugarcane, and cereals, has garnered attention (Santika *et al.*, 2019), apprehension has emerged regarding the use of edible biomass for energy generation due to their significance as carbohydrate sources for human consumption (Panja *et al.*, 2023). To counter this

concern, researchers are actively exploring non-edible biomass alternatives that are suitable for biofuel production (Otoikhian and Amune, 2022). Sugarcane bagasse stands as one such alternative—a residue accounting for approximately a quarter of the total weight after juice extraction 60% to 80% carbohydrates (Sing *et al.*, 2023). However, the fermentation of these carbohydrates remains underutilized, as bagasse is typically either discarded as agricultural waste or incinerated for energy in sugar and ethanol mills. Both disposal methods are, however, environmentally detrimental and inefficient in harnessing the chemical energy latent in the biomass (Zadeh *et al.*, 2020).

To harness sugarcane bagasse's potential as a renewable biofuel feedstock, the fractionation of its constituents and their subsequent conversion into fermentable sugars necessitate consideration. Nonetheless, the presence of substantial lignin content renders bagasse recalcitrant to hydrolysis, prompting the requirement for pretreatment technologies to transform its components into fermentable sugars for biofuel synthesis. Various pretreatment methodologies have emerged and been employed to heighten fermentable sugar yields while minimizing carbohydrate degradation, production of enzyme inhibitors, and generation of toxic byproducts (Alawad and Ibrahim, 2022). These techniques encompass physical, physical-chemical, and biological processes, each wielding distinct mechanisms—such as decreasing cellulose crystallinity and/or polymerization degrees, amplifying accessible surface areas, or selectively extracting hemicellulose and lignin from the lignocellulosic structure (Panja *et al.*, 2023).

An effective pretreatment strategy ought to also curtail carbohydrate degradation, enzyme inhibitor generation, and toxic byproduct formation—facilitating the fermentation of microorganisms. Thus, the employment of experimental design and data analysis surfaces as pivotal practices for optimizing process parameters that govern bioethanol production. Within this context, Response Surface Methodology (RSM) emerges as an array of mathematical and statistical techniques that furnish modeling capabilities. RSM serves as a statistical framework for experimental design, factor effect evaluation, and the determination of optimal conditions yielding desirable responses,

all while minimizing the requisite number of trials. Employing a Central Composite Design (CCD), an empirical second-order polynomial model is devised to optimize the production of bioethanol from sugarcane bagasse (Namnuch *et al.*, 2021). The ongoing trend in global energy consumption underscores the urgency of alternative and renewable energy sources to curtail CO₂ emissions and offset climate change effects. Particularly, within this sphere, biofuels—especially bioethanol—present a propitious avenue, derived from non-edible biomass sources such as sugarcane bagasse (Oyedeji *et al.*, 2021).

1.1 Statement of the Problem

The major problem of the current and upcoming world community is the depletion of energy supply owing to the continuous utilization of resources. It has been predicted in the coming years that fossil fuel could be depleted as a result of dramatic shift to more efficient fuels. Secondly, the use of fossil fuels is increasingly causing depletion of the ozone layer due the continuous release of greenhouse gases which cause global warming. Thus, it is of interest to find non-petroleum-based alternative sources of energy that are clean, renewable and do not conflict with human feeding.

2.0 MATERIALS AND METHODS

2.1. Substrate

The material utilized in this investigation was sugarcane bagasse, procured from local vicinity (Gusau, Nigeria). The initial step involved desiccating 70kg substrate (sugar bagasse) to eliminate water molecules, followed by milling to obtain a finely powdered and fibrous form suitable for subsequent experimentation. Precisely, the biomass underwent chopping and washing stages to eliminate extraneous elements. Subsequently, it was subjected to oven drying at 70°C for a span of three days, followed by pulverization using a hammer mill and screening to achieve a particle size of 1 mm using planetary method. The desiccated samples underwent evaluations for moisture and ash content AOAC, (1990). For the primary constituents of the raw material—cellulose, hemicellulose, and lignin—quantitative analyses were in accordance with the AOAC Official Method 1973. Comprehensive chemical assessments of the biomass were performed both before and after the pretreatment

process, aiming to discern the influence of the pretreatment on the substrate's compositional makeup

2.2 Source and Preparation of Microorganism

The microorganism utilized in this research (*A. flavus*) was isolated, identified, and readied as outlined below: Initially, the microorganism's cultures were cultivated and sustained on Potato Dextrose Agar (PDA) slants. Periodic sub-culturing occurred every 15 days. The cultures were preserved in a refrigerator at a temperature of 4°C, kept on standby for fermentation. Inoculum preparation commenced a 24-day-old *A. flavus* culture. In succinct terms, a small amount of the cultures were introduced into the growth medium, subjected to 48 hours of incubation at 30°C on a rotary shaker. To amass the cells, a centrifugation process was conducted for 48 hours at 200 rpm using benchtop centrifuge. A 0.1% inoculum concentration (calculated on a dry weight to volume basis) was employed for the ensuing fermentation procedure.

2.2 Substrate Pretreatment

The desiccated substrate underwent pretreatment using a NaOH solution according to the ensuing protocol: A quantity of forty grams of finely ground substrate was suspended in 400ml of a 5% w/v NaOH solution, producing a substrate-NaOH suspension. A 10ml sample of this suspension was then moved to a screw-capped Erlenmeyer flask and subjected to a 2-hour heating process in a water bath sustained at 100°C. Post the 2-hour heating interval, the solid residue was retrieved through vacuum filtration, systematically rinsed with water until achieving a neutral pH. Following this, the moisture content was quantified, and the material was preserved within a sealed plastic bag at 4°C for subsequent utilization as the substrate in the cellulose saccharification experiment. Notably, prior to enzyme digestion, the pretreated substrate was intentionally not desiccated. This measure aimed to prevent pore collapse within the biomass's microstructure, which, if present, could result in a reduction in the enzymatic glucose release during the cellulose saccharification process.

2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in accordance with the procedure of albeit with minor adjustments. The hydrolysis process utilized a 50 mM citrate buffer at pH 4.8, augmented with 0.02% sodium azide. Conventional hydrolysis kinetics were executed at a solid load of 10%, employing enzyme loads of 10, 20, and 40 FPU/g of bagasse, all within a reaction volume of 30 mL. Following hydrolysis, samples from the resulting hydrolysate were subjected to centrifugation at 400 rpm and 4°C for 10 minutes. The resultant liquid supernatant was collected for the purpose of quantifying glucose content. The conversion of biomass (X) was assessed using the subsequent formula (Equation 1).

$$X = \left(\frac{Sg \times VH}{Wdry \text{ bagasse} \times Fg} \right) 0.9 \times 100\% \dots \dots \dots [1]$$

Considering the quantity of soluble sugars within the aqueous phase post-hydrolysis (Sg, representing glucose equivalent concentration), the volume of the solution used for hydrolysis (VH), and a correction factor for hydration set at 0.9 to account for the additional water molecules introduced during the hydrolysis process, alongside the original glucose equivalent content within the dry lignocellulosic sample prior to hydrolysis (W, denoting the weight of the desiccated lignocellulosic sample), and the fraction of cellulose present within the lignocellulosic sample (Fg) – all factors were taken into consideration.

2.3.1 Determination of Reducing Sugar

The quantification of reducing sugar content within the hydrolysates was conducted according to the method of Miller (1959) Specifically, equimolar volumes (3ml each) of 3,5-dinitrosalicylic acid (DNS) reagent and the hydrolysate were introduced into separate test tubes. Thorough mixing was achieved by shaking the test tubes, which were then subjected to boiling water (100°C) for a span of 10 minutes, leading to the development of a reddish-brown hue. Additionally, a 40% solution of potassium sodium tartrate was introduced to stabilize the reactions, followed by cooling to room temperature via running tap water. Subsequent spectrophotometric analysis at a wavelength of 490nm facilitated

absorbance measurement. The concentrations of reducing sugars were subsequently determined via extrapolation, utilizing a well-prepared standard curve of known glucose concentrations.

2.3.2 Sugar Fermentation

The fermentation process for the hydrolyzed samples was carried out following the procedure described by Brooks (2008).

Each fermentation involves introducing 50ml of hydrolysate into 100ml conical flasks, which were then subjected to autoclaving at 120°C for 15minutes. Subsequently, the flasks were allowed to cool before the inoculation step. Various concentrations of inoculum were then introduced into the autoclaved hydrolysates in aseptic conditions. The quantification of bioethanol production entailed treating 4ml of fermented samples with 2ml of a chromium reagent. This mixture was subsequently incubated for 1hour at 37°C, and absorbance was measured spectrophotometrically at 588nm.

To ascertain the percentage yield of bioethanol, both the initial and final content of reducing sugars in the medium were documented.

Equation 2 was then utilized to compute the bioethanol yield.

$$\text{Bioethanol yield (\%)} = \frac{\text{Ethanol produced} \times 100}{\text{Reducing sugar Utilized}} [2]$$

2.4 Box-Behnken Experimental Design

In this study, the investigation into the factors governing the rate of bioethanol production from sugarcane bagasse employed the Box-Behnken design (BBD). In this design, four crucial operational parameters—namely, inoculum concentration, pH, temperature, and fermentation time—were selected. The Box-Behnken design entailed manipulating these factors across various levels (Table 1), generating a design matrix that incorporated randomized operational variable settings. The impact of these parameters on the fermentation process for bioethanol production was further assessed using Analysis of Variance (ANOVA) on statistical package for social science (SPSS), aiding in pinpointing the factors that exert a substantial influence on the response

Table 1: Factors Used in Response Surface Optimization

Factors	Name	Units	Minimum	Maximum
A	Temperature	°C	30.00	50.00
B	Fermentation Time	hours	35.00	95.00
C	Inoculum Concentration	% v/v	5.00	20.00
D	pH		3.00	7.00

The matrix encompassing the independent variables and the outcomes of 29 runs conducted in triplicates, both in terms of experimental and projected bioethanol yield, is detailed in Table 2. It's noticeable that a significant correspondence

existed between the experimental and anticipated bioethanol concentrations under different conditions. This demonstrates the remarkable precision of the response surface model that was formulated within this research

Table 2: Box-Behnken Design Matrix

Run	Temperature oC	pH	Inoculum Concentration % v/v	Fermentation Time hours	Bioethanol Yield %
1	40	5	12.5	65	55
2	30	7	12.5	65	43
3	30	5	5.00	65	37
4	30	5	20.0	65	58
5	40	5	12.5	65	72
6	50	5	12.5	95	83
7	50	5	5.00	65	77

8	50	5	20.0	65	64
9	40	3	5.00	65	45
10	40	3	20.0	65	57
11	50	7	12.5	65	88
12	30	5	12.5	35	33
13	30	5	12.5	95	81
14	40	3	12.5	95	90
15	40	5	20.0	35	44
16	30	3	12.5	65	59
17	40	7	20.0	65	62
18	40	5	5.00	35	32
19	40	7	12.5	95	92
20	40	3	12.5	35	70
21	40	5	12.5	65	65
22	40	5	5.00	95	85
23	40	7	5.00	65	65
24	40	7	12.5	35	31
25	40	5	12.5	65	54
26	40	5	20.0	95	95
27	40	5	12.5	65	68
28	50	5	12.5	35	51
29	50	3	12.5	65	63

2.4.1 Regression Model and its Validation

The primary focus of this investigation revolves around the effective quantity of bioethanol generated, which signifies the real yield of bioethanol in relation to the initial substrate's (sugarcane bagasse) total sugar content concentration. The comprehensive design matrix incorporating experimental results of bioethanol yield (%) is outlined in Table 2. Through the utilization of BBD and experimental observations, the ensuing second-order quadratic model equation, depicting the impact of the varied factors under scrutiny on the process yield, was derived.

$$\text{Bioethanol Yield} = 62.8 + 9.58A - 0.25B + 3.25C + 22.08D + 10.25AB - 8.50AC - 4.00AD - 3.75BD + 10.25BC - 0.5CD - 1.98A^2 + 1.53B^2 - 3.98C^2 + 4.27D^2$$

The equation defines Y as the bioethanol yield %, where a positive coefficient signifies a

cooperative influence, while a negative coefficient suggests an antagonistic effect. The model's outcomes reveal that, within the investigated experimental range, the fermentation time exerts the most prominent positive influence on the

process, followed by temperature, inoculum concentration, and pH, in a descending sequence. Although initial pH yields a minor positive effect on bioethanol yield (%), its quadratic impact manifests the most significant negative influence on the fermentation process, succeeded by the negative quadratic effect of incubation period. Regarding quadratic effects, the sequence of positive impacts on fermentation consists of the inoculum concentration, incubation temperature, and mixing rate, in decreasing order. Moreover, the positive interactive effects of the studied parameters are ranked as follows: mixing rate and incubation period > initial molasses concentration and incubation period > initial pH and mixing rate > incubation period and temperature > initial molasses concentration and incubation temperature. Notably, the significance of the model's validity was assessed through F -test and evaluated for statistical importance using analysis of variance (ANOVA), Table 3. The model (equation 4) proved to be statistically significant at a 95% confidence level, displaying a F -value of 7.57 and a probability P value of 0.0003—implying less than a 0.003% likelihood of error

originating from noise. The determination coefficients R^2 and R^2_{adj} , gauging the reliability of model fit, stood at 0.953 and 0.920, respectively. This signified approximately 95.3% of variance attributed to the variables, validating the model's significance. A mere 4.7% of total variations remained unaccounted for by the model (equation 4), attesting to the strong fit of the predicted model with the experimental data. Further evidence of the regression model's adequacy was witnessed through the commendable alignment between

experimental and predicted response variable values, as displayed in Table 2. The "Adeq Precision" parameter, gauging the signal-to-noise ratio, yielded a favorable ratio of 17.1 for model (equation 4), indicating adequate signal strength. This model's reliability is upheld, warranting its utilization for navigating the design space. Notably, the standard deviation (SD) and coefficient of variance, representing model equation (4), were minimal, registering 2.87 and 12.8, respectively

Table 3: Results of Analysis of Variance for the Quadratic Model

Source	Sum of square	df	Mean Square	F-Value	P-value
Model	8654.83	14	618.20	7.57	0.0003
A-Temperature	1102.08	1	1102.08	13.50	0.0025
B-pH	0.7500	1	0.7500	0.0092	0.9250
C-Inoculum Concentration	126.75	1	126.75	1.55	0.2332
D-Fermentation Time	5852.08	1	5852.08	71.68	< 0.0001
AB	420.25	1	420.25	5.15	0.0396
AC	289.00	1	289.00	3.54	0.0809
AD	64.00	1	64.00	0.7839	0.3909
BC	56.25	1	56.25	0.6890	0.4204
BD	420.25	1	420.25	5.15	0.0396
CD	1.00	1	1.00	0.0122	0.9134
A ²	25.52	1	25.52	0.3125	0.5850
B ²	14.92	1	14.92	0.1828	0.6755
C ²	102.92	1	102.92	1.26	0.2804
D ²	118.08	1	118.08	1.45	0.2491
Residual	1142.97	14	81.64		
Lack of Fit	888.17	10	88.82	1.39	0.4008
Pure Error	254.80	4	63.70		
Cor Total	9797.79	28			

2.4.2 Diagnostic Analysis of the Regression Model

The model's effectiveness is evident from the comparison of predicted and experimental bioethanol yield results depicted in Figure (a), revealing a substantial correlation coefficient ($R^2 = 0.74$). This signifies a favorable agreement between predicted and actual values. Thus, the data harmoniously align with the models, offering a reliable estimation of the response within the

explored experimental scope. In Figure 1(b), the residual distribution plot is presented, illustrating the deviation between calculated and observed response variable values against predicted response. Notably, the quality of fit remains robust, as the residual distribution doesn't exhibit a discernible pattern concerning predicted response values. This observation underscores the quadratic model's adequacy in representing the bioethanol % yield consistently across the span of the studied experimental range.

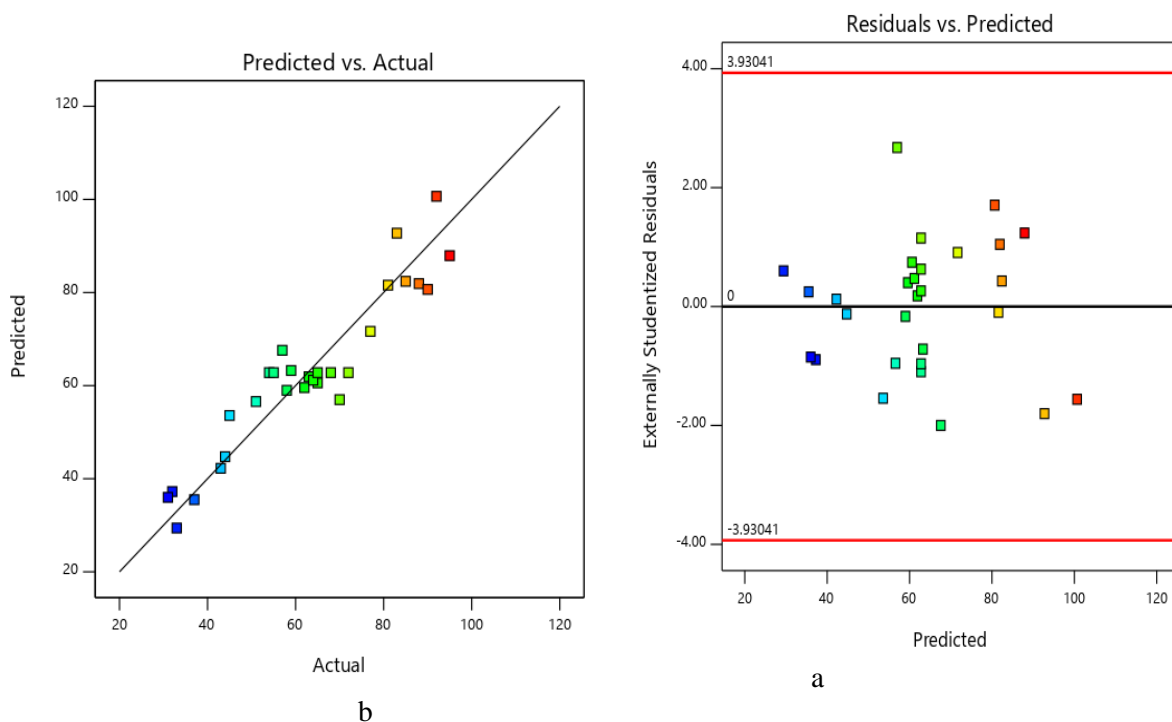


Figure 1a&b: Diagnostic Plot of the Box-Behnken Model

3. Optimization of the Fermentation Process

Three-dimensional response surfaces were generated using the predicted model equation to explore the interplay among variables and determine optimal conditions for achieving maximum bioethanol % yield. The interaction between Temperature and pH is apparent from the RSM and contour plots shown in Figure 1(a). As initial pH rises, bioethanol yield increases until reaching its zenith around pH 5, subsequently decreasing with further increases in pH. Meanwhile, an augmentation in inoculum concentration correlates with an increased bioethanol yield, with the highest yield observed at pH 5 and 20% inoculum concentration. The elliptical pattern in Figure 1(b) signifies the negative interactive effect of initial pH and incubation period on bioethanol yield. In this context, the yield escalates as fermentation time and initial pH increase, peaking at 94% at pH 5 within the 60–90-hour timeframe. Nevertheless, this trend reverses when initial pH and fermentation time are raised further. The RSM and contour plots in the supplementary materials depict a positive interactive effect between fermentation time and inoculum concentration. During the 40–

60-hour interval, an increase in fermentation time and inoculum concentration results in enhanced bioethanol yield, yet an extended fermentation period diminishes the yield. However, the plots suggest that significantly higher fermentation times (≥ 60 hours) do not lead to substantial yield improvements. Figure 1(d) highlights the positive interactive impact of inoculum concentration and pH. An increase in both factors corresponds to heightened bioethanol yield, peaking at approximately 44% with 25% initial inoculum concentration at 35°C.

Numerous studies, including Sun *et al.*, 2023, emphasize the significance of pH in microbial growth and product formation during fermentation. Notably, Misono and Yamaguchi (2018) noted an optimum pH of 5 for molasses fermentation, while Yadav *et al.* 2020 identified the pH range of 4.5–5 as optimal for alcohol productivity. Indicated that the yeast strain K211 exhibited peak cell viability and ethanol productivity at 25% (w/v) molasses concentration and 35°C. Santika *et al.*, 2019, highlighted a pH of 5.3 and a 24-hour incubation time as optimal for bioethanol production from molasses. They also pointed out that longer incubation periods could

lead to reduced bioethanol production due to sugar depletion, ethanol oxidation, and organic acid formation. Moreover, Dombek and Ingram elucidated that ethanol accumulation in fermentation broth could deactivate alcohol-producing enzymes.

Optimization was executed using Design Expert 6.0.7 software (Stat-Ease Inc., Minneapolis, USA) to establish the optimum values for the five parameters affecting sugarcane molasses fermentation by *Pichia veronae* strain HSC-22. The objective was to maximize both bioethanol production (g/L) and bioethanol yield. The software's optimization step involved defining desired goals for each fermentation parameter

within the studied range to achieve the highest performance. A desirability function amalgamated individual desirability values into a single number for optimization. Accordingly, the optimal conditions for maximum bioethanol production, with a bioethanol yield of 95%, were determined as follows: pH 5, 20% inoculum concentration, 45°C, and 60 hours, yielding a desirability function value of 1. Experimental results under these conditions yielded 92 g/L bioethanol production with a bioethanol yield of 43.57%. This attests to the reliability and effectiveness of the Box-Behnken Design of experiments for optimizing the bioethanol fermentation process using Sugarcane bagasse and *Aspergillus flavus*

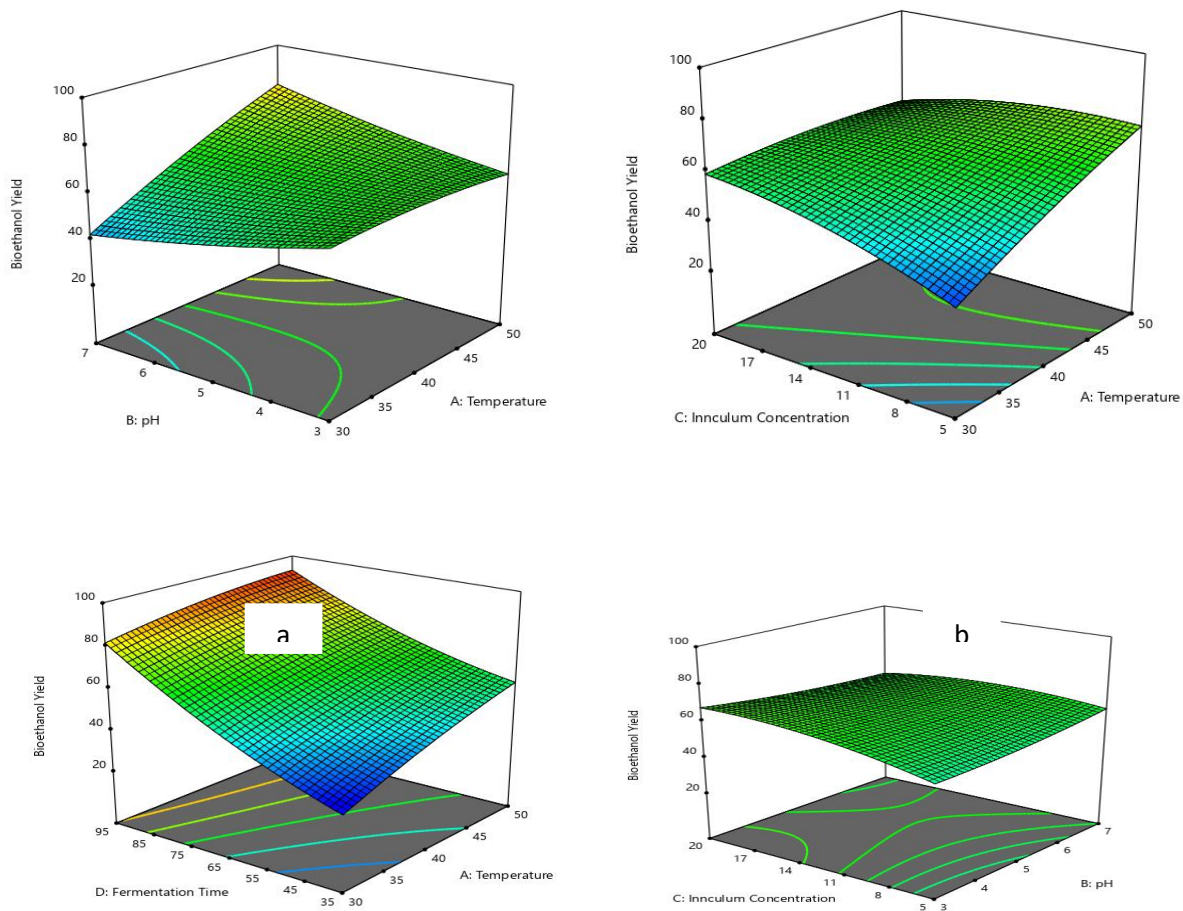


Figure 2 a,b,c&d: Three Dimensional Response Surface Plots of the Box-Behnken Design

Conclusion

The application of the Box-Behnken Design (BBD) in experiments proved effective in

modeling ethanol productivity during batch fermentation of sugarcane bagasse using *Aspergillus flavus*. Leveraging the developed quadratic model, along with response surfaces and contour plots, facilitated the examination of how bioethanol yield varies based on modifications in process variables such as pH, molasses concentration, temperature, mixing rate, and time.

Moreover, the quest for maximizing bioethanol yield became readily attainable through these tools. Ongoing efforts within the research group involve delving into the impact of distinct sugarcane bagasse constituents and secondary fermentation byproducts on the fermentation process and resultant bioethanol yield.

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